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SM934, a water-soluble derivative of arteminisin, exerts immunosuppressive functions *in vitro* and *in vivo*

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ABSTRACT

In the present study, we investigated the immunosuppressive effects and underlying mechanisms of β-aminoarteether maleate (SM934), a derivative of artemisinin, against T cell activation *in vitro* and *in vivo*. *In vitro*, SM934 significantly inhibited the proliferation of splenocytes induced by concanavalin A (Con A), lipopolysaccharide (LPS), mixed lymphocyte reaction (MLR), and anti-CD3 plus anti-CD28 (anti-CD3/28). SM934 significantly inhibited interferon (IFN)-γ production and $CD4^+$ T cell division stimulated by anti-CD3/28. SM934 also promoted apoptosis of $CD69^+$ population in $CD4^+$ T cells stimulated by anti-CD3/28. Furthermore, SM934 inhibited interleukin (IL)-2 mediated proliferation and survival through blocking Akt phosphorylation in activated T cells. In ovalbumin (OVA)-immunized mice, oral administration of SM934 suppressed OVA-specific T cell proliferation and IFN-γ production. SM934 treatment also significantly inhibited the sheep red blood cell (SRBC)-induced delayed type hypersensitivity (DTH) reactions in mice. Taken together, SM934 showed potent immunosuppressive activities *in vitro* and *in vivo*. Our results demonstrated that SM934 might be a potential therapeutic agent for immune-related diseases.

1. Introduction

Derivatives of artemisinin are currently recommended as first-line antimalarial agent [1]. Besides having antimalarial activity, these drugs also exhibit potent immunosuppressive effects. In China, artemisinin derivatives have shown promising results when tested for treatment of autoimmune diseases, such as systemic lupus erythematosus (SLE) and allergic contact dermatitis [2–4]. However, the efficacy of clinically relevant artemisinin derivatives is limited, and their immunosuppressive mechanisms are still obscure. Therefore, we synthesized a series of new water-soluble and oil-soluble artemisinin derivatives and screened for a promising compound with potent immunosuppressive activity [5,6]. SM934, β-aminoarteether maleate (Fig. 1), was identified as a promising compound with higher bioavailability and drug likeability. In this study, we investigated the immunosuppressive effects of SM934 on T cell activation, both *in vitro* and *in vivo*, and explored its potential mode of action.

T cells play a pivotal role in acquired immune reaction and have been implicated in mediating many aspects of autoimmune diseases [7]. Firstly, TCR cross-linking drives T cells from G0 to G1 transition and subsequent secretion of T cell growth factor IL-2 and expression of high-affinity receptor IL-2Rα chain (CD25); secondly, through autocrine/paracrine proliferative loop, IL-2 induces clone expansion and maintains survival of activated T cells; thirdly, once there is successful clearance of the pathogen/autoantigen, the stimulus for cytokines production is lost and the majority of activated T cells enter apoptosis [8,9]. Whereas, in the circumstance of autoimmune diseases, persistent presence of autoantigen provides the survival signals for activated autoreactive lymphocytes to avoid apoptosis, which results in sustained tissue injury [10,11]. Abrogating IL-2 production by Cyclosporin A (CsA) has revolutionized the field of immune therapy [12]. The immunosuppressive action of CsA involves initial binding to cyclophilin, leading to reduced IL-2 production as part of the signal transduction pathway for the activation of T cells.

Actually, IL-2 often acts as a "double-edged sword" in immune responses. On one hand, IL-2 is a growth factor for T cells and plays important roles in the immune response. On the other hand, IL-2 is also critical for the development of immunosuppressive regulatory T cells, and IL-2 deficient mice develop severe autoimmune disorders [13,14]. And IL-2 secretion by T cells is decreased in some autoimmune diseases, especially systemic lupus erythematosus [15,16]. Thus, drugs affecting

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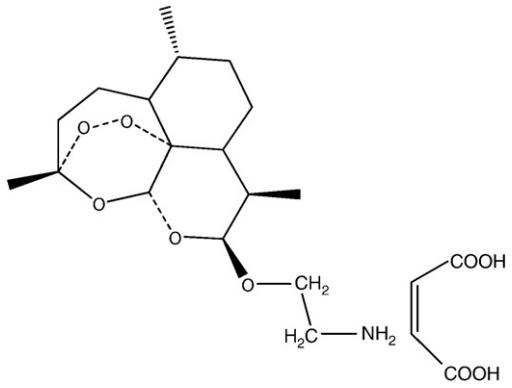

Fig. 1. Chemical structure of SM934.

IL-2-dependent T cell activation rather than IL-2 production should be of particular interest for their potential clinical uses.

To gain insight into the molecular mechanism of action of SM934, we investigated the effects of SM934 on TCR cross-linking induced proliferation and cytokine production in T cells, which were mimicked by anti-CD3/28 stimulation. The results showed that SM934 significantly inhibited T cell proliferation without much influence on IL-2 production. Further studies revealed that SM934 inhibited IL-2 mediated proliferation and survival through blocking Akt phosphorylation in activated T cells. Using OVA-immunized mice and SRBC-induced DTH reaction, we demonstrated that SM934 also had the potent immunosuppressive effects *in vivo*. These results indicated that SM934 possessed potent immunosuppressive activities *in vitro* and *in vivo*. SM934 might to be a novel immunosuppressive agent.

2. Materials and methods

2.1. SM934

SM934, β-aminoarteether maleate, was synthesized from β-hydroxyarteether at Shanghai Institute of Materia Medica. Before use, SM934 was dissolved in phosphate buffered saline (PBS) or saline as a stock solution, and stored at −20 ºC. The chemical structure is shown in Fig. 1.

2.2. Mice

Female BALB/c and C57BL/6 mice (6 to 8-week old) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Certificate No.2002-0010). The mice were housed under specific pathogen-free conditions. All mice were fed standard laboratory chow and water *ad libitum*. The environment was maintained at 22 ± 1 °C with a 12-h light and dark cycle. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica.

2.3. Splenocyte preparation

Mice were sacrificed and their spleens were removed aseptically. The spleens were pressed against the bottom of the petri dish with the plunger of a 6-ml syringe. A single cell suspension was prepared and cell debris and clumps were removed. Erythrocytes were lysed with Tris-buffered ammonium chloride (0.155 M NH_4Cl and 16.5 mM Tris, pH 7.2). Cells were washed and resuspended in RPMI 1640 media (containing 10% FBS) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were counted by trypan blue exclusion. The viability of splenocytes was consistently > 99%.

2.4. MTT assay

Cytotoxicity was assessed by the MTT assay. Briefly, splenocytes were cultured in triplicate for 48 h with SM934. The cells cultured with media alone were used as controls. MTT (5 mg/ml) reagent was added 4 h before the end of culture, and then cells were lysed with 10% sodium dodecyl sulfate (SDS), 50% *N*, N-dimethyl formamide, pH 7.2. O.D. values were read at 570 nm, and the percentage of cell death was calculated. Experiments were conducted at least three times.

2.5. Con A and LPS-induced proliferation assay

Splenocytes were cultured in triplicate for 48 h with 5 μg/ml of Con A or 10 μg/ml of LPS plus SM934. Cells were pulsed with 0.5 μCi/ well of [^{3}H]thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, PerkinElmer Life Sciences, Boston, MA). Experiments were conducted at least three times.

2.6. Mixed lymphocyte reaction assay

BALB/c splenocytes (3×10^5 cells/well, stimulator cells) were pretreated with 30 Gy γ-irradiation (Gammacell 3000, Ottawa, ON, Canada) and then co-cultured in triplicate with C57BL/6 splenocytes (3×10^5 cells/well, responder cells) in the presence or absence of SM934. After 72 h, cells were pulsed with 1 μCi/well of [^{3}H]-thymidine and incubated for another 24 h. Cells were harvested onto glass fiber filters and incorporated radioactivity was counted using a Beta Scintillation Counter.

To determine cytokine levels, the cultures were incubated for 72 h and the supernatants were collected by centrifugation at 300 *g* for 5 min and stored at −20 °C. Mouse IL-2 and IFN-γ level in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences and Pharmingen, San Diego, CA, USA) following the manufacturer's instruction. Experiments were conducted at least three times.

2.7. $CD4^+$ T cell isolation

Purified $CD4^+$ T cells from BALB/c mice were prepared by using immunomagnetic negative selection to deplete $CD8^+$ T cells, B-cells, NK T cells and I-A^+ APCs as described previously with slight modification [17]. Lymphocytes were reacted with anti-I-Ad/b, anti-B220, anti-Mac1, anti-CD8, and anti-NK1.1 (mAbs used here were all purified from ascites using agarose-conjugated-protein G column in our lab) and then incubated with magnetic particles bound to goat anti-rat and goat anti-mouse Ig (Advanced Magnetics). Purity of the resulting $CD4^+$ T cell populations was examined by FACS Calibur (Becton Dickinson, San Jose, CA, USA), and was consistently > 93%.

2.8. Anti-CD3/28 mAb mediated primary $CD4^+$ T cells activation

Primary $CD4^+$ T cells (2×10^5 cells/well) were cultured with anti-CD28 mAb (1 μg/ml) in 96-well flat-bottom plates coated with anti-CD3 mAb (5 μg/ml). SM934 (1 and 10 μM) was added simultaneously. $CD4^+$ T cells were cultured for 48 h to assess [^{3}H]thymidine incorporation and to determine IL-2 and IFN-γ productions. For activated marker expression and apoptosis determination, cells were cultured for 24 h. Experiments were conducted at least three times.